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Article

Fates of Antibiotic Resistance Genes in the Gut Microbiome from Different Soil Fauna under Long-Term Fertilization

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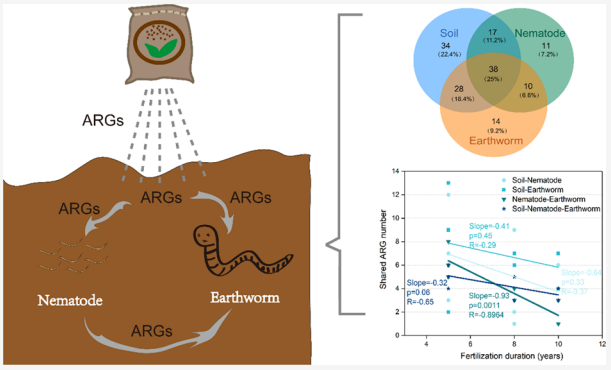
ABSTRACT: Applying organic fertilizers has been well documented to facilitate the dissemination of antibiotic resistance genes (ARGs) in soil ecosystems. However, the role of soil fauna in this process has been seldom addressed, which hampers our ability to predict the fate of and to manage the spread of ARGs. Here, using high-throughput quantitative polymerase chain reaction (HT-qPCR), we examined the effect of long-term (5-, 8-, and 10-year) fertilization treatments (control, inorganic fertilizers, and mixed fertilizers) on the transfer of ARGs between soil, nematodes, and earthworms. We found distinct fates for ARGs in the nematodes and earthworms, with the former having higher enriched levels of ARGs than the latter. Fertilization impacted the number and abundance of ARGs in soil, and fertilization duration altered the composition of ARGs. Shared ARGs among soil, nematodes, and earthworm guts supported by a fast expectation-maximization microbial source tracking analysis demonstrated the trophic transfer potential of ARGs through this short soil food chain. The transfer of ARGs was reduced by fertilization duration, which was mainly ascribed to the reduction of ARGs in the earthworm gut microbiota. This study identified the transfer of ARGs in the soil-nematode-earthworm food chain as a potential mechanism for a wider dissemination of ARGs in the soil ecosystem.



INTRODUCTION

It is recognized that antibiotic resistance genes (ARGs) increasingly pose a potential risk to public health and are a global pollutant.[1−3] To date, ARGs associated with wastewater,[4] sludge,[5] and manure[6] have received the greatest research focus. Through both wastewater irrigation and organic fertilizer application, ARGs have been disseminated to agricultural soils and subsequently transferred to both the wider environment and crop plants,[7,8] with subsequent detection in drinking water[9] and salad.[10]

Recently, several studies have investigated the effect of fertilization on ARGs in soil microbiota and compared the different effects of mineral and organic fertilizers.[11,12] A farm study (Hunan, China) using high-throughput quantitative polymerase chain reaction (HT-qPCR) indicated that a 25-year application of mineral fertilizers had only a moderate effect on soil ARG profiles, but pig manure significantly altered the soil ARG composition with the introduction of new ARGs into the soil.[6] However, another study based on qPCR in a Danish farmland demonstrated that the application of pig manure containing tetracycline elevated the tetracycline resistance level in soil microbiota, but the resistance level declined compared to that of controls over time (8 months).[13] Furthermore, manure application did not show a significant enrichment of ARGs in soil microbiota from a 26-year experimental field (Yingtan, China).[14] Hence, the addition of ARGs in the field might be attenuated with time, but the mechanism for that is unclear.

Soil fauna is highly diverse, with for example >10,000 protozoans, 10−100 nematodes, mites, and collembola per gram soil.[14] In addition, macrofauna typically represents 100−500 earthworms per m^2 of soil.[15] Soil fauna plays a key role in soil functions and ecological processes[16] and harbors diverse soil-derived microbiota, particularly in their guts.[17,18] Thus, the gut microbiome in soil fauna may reflect environmental perturbations of the adjacent soil.[19] Multiple studies have demonstrated that soil fauna gut microbiome could be enriched in ARGs, exposed to antibiotics, and be a reservoir for ARGs.[11,12] Also, ARGs in swine manure were reported to have been attenuated by housefly gut microbiota.[20] However,

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the fate of ARGs in the gut microbiome of different soil fauna is ill-defined.

ARGs can be dispersed belowground through different trophic levels of the soil food web.[21] As key members of the soil food web, nematodes and earthworms participate in many major soil processes (e.g., litter decomposition,[22] formation of soil aggregates,[23] maintenance of biodiversity,[24] energy transfer,[25] and material circulation[26] via metabolism and food ingestion).[27] Nematodes are the most abundant phyla[28] on earth and are multitrophic[29] as well as a dietary source for earthworms.[16,30−33] Predation of earthworms on nematodes not only directly impacts the nematode community structure[34,35] but also potentially further accelerates the transfer of ARGs through the food chain similar to that reported for the collembolan-predatory mite food chain.[21] However, detailed knowledge of the interaction between nematodes and earthworms and the effect on spread of soil antibiotic resistance is unknown.

In this study, Illumina sequencing of the 16S rRNA gene and HT-qPCR were used to characterize the microbiome and associated ARGs in soil, nematodes, and earthworm guts. We hypothesize that (1) the fate of ARGs is different in nematodes and earthworm guts due to different intestinal microenvironments; (2) ARG enrichment would occur in the mixed fertilizer treatment, which included organic fertilizers; and (3) the abundance of ARGs in soil resistomes may be attenuated over time by the transmission of ARGs along a short soil food chain.

MATERIALS AND METHODS

Sample Collection. Samples were collected during March 2018 from a long-term monitoring station managed by the Fuyang Agricultural Bureau (30° 04′ N, 119° 57′ E), Zhejiang Province, China. Three sampling sites had fertilizers applied each year since either 2008, 2010, and 2013, representing 10, 8, and 5 years of fertilization, respectively. Three different fertilization treatments: control (C: without fertilizers), inorganic fertilizers (IF: N, P_2O_5, and K_2O), and mixed fertilizers (MF: N, P_2O_5, K_2O, and commercial organic fertilizers) were applied at each sampling site, and the fertilizer application rates are reported elsewhere.[36] Three subsamples were collected by mixing three individual cores collected using soil corers with a diameter of 38 mm (TC-601-B2) from each sampling site, producing 3 composite replicates per site. Soil samples used for nematode isolation and DNA extraction were stored at 4 and −20 °C, respectively. In brief, the nematodes were isolated using a Baermann funnel[37] and were individually picked out under a dissecting microscope (SMZ-168) into absolute ethanol according to morphological characteristics (body size and color),[11] and earthworms were hand-picked and immediately introduced into absolute ethanol.[38] These samples were stored at 4 °C prior to processing.

DNA Extraction. For each sample, randomly selected earthworm adults of approximately the same body size were classified according to their species level by visual examination according to the position of the male genital orifice on the clitellum and dissected with aseptic forceps and needles after being washed several times with sterile phosphate buffer solution.[38] Earthworms were identified using molecular approaches to their species level using Cytochrome OxidaseI-barcode gene sequencing,[18] and two species of earthworms (*Eisenia foetida* and *Pheretima guillelmi*) were identified. *E. foetida*, an epigeic species, was present in all treatments.[39,40] *P. guillelmi*, also an epigeic species, was only collected in 18 samples except the 5-year fertilization treatment. Thus, *E. foetida* samples were chosen for DNA extraction from guts. All treatments had three replicates. Guts were collected from five individual earthworms for each replicate sample. Also, twenty nematodes were randomly selected for each replicate sample, placed into 2% sodium hypochlorite solution for 10 s to remove any bacteria adhering to the nematode cuticle, and rinsed four times with aseptic phosphate buffer.[11] Bacterial removal efficiency was checked by spreading the final wash on an LB (Luria−Bertani) agar plate with no observation of any colonies. Thereafter, earthworm guts and nematodes were transferred into a 1.5 mL centrifuge tube via sterile nippers under aseptic conditions and smashed by small crushing pestles. DNA was extracted with a DNeasy blood & tissue kit (QIAGEN, Hilden, Germany) based on the manufacturer's guidelines. Soil DNA was extracted from 0.5 g of soil using a FastDNA Spin Kit (MP Biomedicals, USA) following the manufacturer's instructions. Extracted DNA was stored at −20 °C.

16S rRNA Amplicon Sequencing. The 16S rRNA gene was amplified with primers 515F and 806R.[17,36] Amplification products were submitted for high-throughput sequencing on the Illumina Hiseq2500 platform (Novogene, China).[36] Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.1) was used to analyze the sequencing data.[41] Open-reference operational taxonomic units (OTUs) were identified at 97% sequence similarity using UCLUST clustering.[42] Representative sequences of each OTU were assigned to taxonomic lineages using the PyNAST and RDP classifier.[43] The sequencing process is outlined in detail under the Methods section of the Supporting Information. Sequencing data were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) with Bioproject accession numbers PRJNA530389 and PRJNA540294.

HT-qPCR for ARG Analysis. Relative abundance and diversity of ARGs in all samples were detected using the HT-qPCR via the Wafergen SmartChip real-time PCR system (Warfergen, USA). Data were analyzed with SmartChip qPCR software (V 2.7.0.1). Amplification was regarded as positive if all three technological replicates were observed, and a threshold cycle (C_t) of 31 was set as the detection limit.[4,44] HT-qPCR for ARG analysis is illustrated in detail under the Methods section of the Supporting Information.

Statistical Analysis. Averages and standard deviations (SD) of microbial and ARG abundance data were calculated using Excel 2016 (Microsoft Office 2016, Microsoft, USA), and one-way ANOVA was carried out using SPSS 22 (SPSS, Inc., Chicago, IL). Pie graphs, column, box-plot, and scatter diagrams were generated using Origin 2017. Alpha diversity of OTUs and ARGs was determined by the Shannon index.[45] Discrepancies in the microbial communities among samples were assessed by principal coordinate analysis (PCoA). PCoA, redundancy analysis (RDA), and Procrustes tests were carried out using R version 3.4.1 with "vegan" package[46] and were used to identify the overall pattern of ARGs across samples and the relationships between ARGs and microbiome. Bipartite network analysis of ARGs characterized in soil, nematodes, and earthworm guts and a co-occurrence network analysis between ARGs and microbial taxa were performed using Gephi 0.9.2.[47] Venn diagrams were generated using Venny 2.1.0.[48] Linear fitting within Origin assessed the correlation between shared

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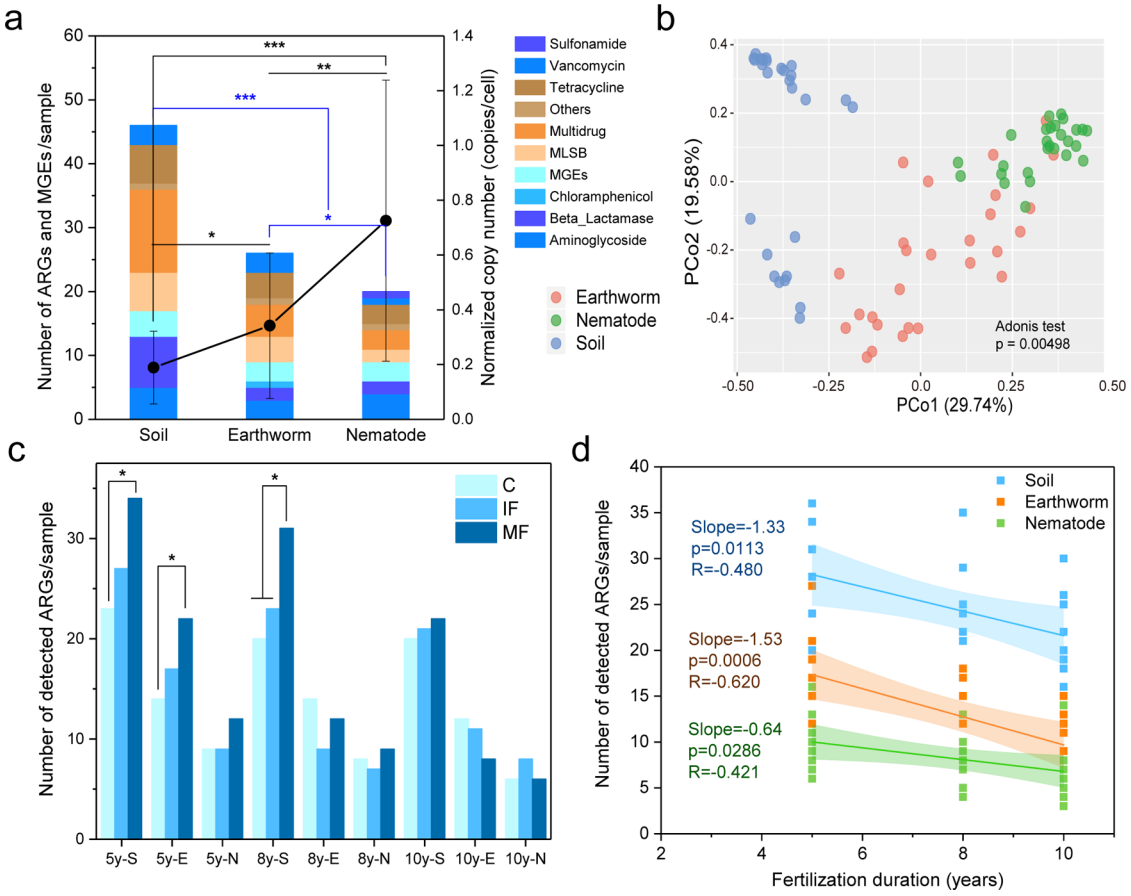

Figure 1. (a) Colored bars and the black line showing the number of each type of ARG and MGE (mean, $n = 27$) and the normalized copy number of ARGs in soil, nematodes, and earthworm guts. ARGs are classified according to their recognized resistance groups. Significant differences in the number of ARGs (ANOVA, Duncan test) and normalized copy numbers (ANOVA, Games-Howell test) between soil, nematodes, and earthworm guts are shown by "*", "**", and "***", which represent $p < 0.05$, 0.01, and 0.001, respectively. (b) Principal coordinate analysis (PCoA) of ARGs' relative abundance in soil, nematodes, and earthworm guts distinguished by different colors (Adonis test, $p < 0.05$). (c) Number of detected ARGs in different fertilization treatments with the control (C), inorganic fertilizer (IF), and mixed fertilizer (MF). Soil, earthworm guts, and nematodes are represented by "S", "E", and "N" and duration of fertilization by "5y", "8y", and "10y", respectively. Significances between fertilization treatments are shown by "*" ($p < 0.05$; ANOVA, Duncan test). (d) Linear regression analysis of duration of fertilization and the total ARG number for soil, nematodes, and earthworm guts. The regression coefficients are represented by the slope of the line, and the linear dependences are depicted by Pearson correlation "R".

ARGs and fertilization duration, and Pearson correlations and associated significance were conducted in SPSS 22. PERMANOVA (Adonis) tests were conducted to display the dissimilarity between treatments based on Bray−Curtis distances. Fast expectation-maximization microbial source tracking (FEAST), a Bayesian classifier method, was used for identifying the source of bacterial communities and followed the guidelines published at https://github.com/cozygene/FEAST.[49] Structural equation models (SEMs) were established using AMOS 21 (SPSS, Inc., Chicago, IL) based on maximum-likelihood estimation to characterize the relationship between fertilization duration, fertilization treatment, soil properties, diversity of earthworm gut microbiota, ARGs in soil, nematode, and earthworm gut microbiome, and MGEs in earthworm gut microbiota. Goodness of fit for the model was tested by multiple criteria.[50] Establishment of the model is described in detail under the Methods section of the Supporting Information.

RESULTS

Characteristics of ARGs in Soil, Nematodes, and Earthworm Guts. A total of 162 ARGs and 9 MGEs were detected across all samples. Detected ARGs were classified into 9 categories: aminoglycoside, beta_lactamase, chloramphenicol, macrolide-lincosamide-streptogramin B (MLSB), multidrug, tetracycline, vancomycin, sulfonamide, and others (Figure 1a). Diversity (Shannon index) of ARGs showed no obvious distinction among soil, earthworm guts, and nematodes (Figure S1), and the number of ARGs exhibited a decrease along soil, earthworm guts, and nematodes (Figure 1a, ANOVA, $p < 0.05$), but the numbers of MGEs in those were similar (Figure 1a). The normalized copy number of ARGs successively increased from soil and earthworm guts to nematodes (Figure 1a, ANOVA, $p < 0.05$). The composition of ARGs detected from soil, nematodes, and earthworm guts was distinct (Figure 1b, PERMANOVA test, $p < 0.05$).

Effects of Fertilization on ARGs in Soil, Nematode, and Earthworm Gut Microbiome. Mixed fertilizers significantly increased the number of detected ARGs in soil (Figure 1c, $p < 0.05$) compared to the control (5- and 8-year

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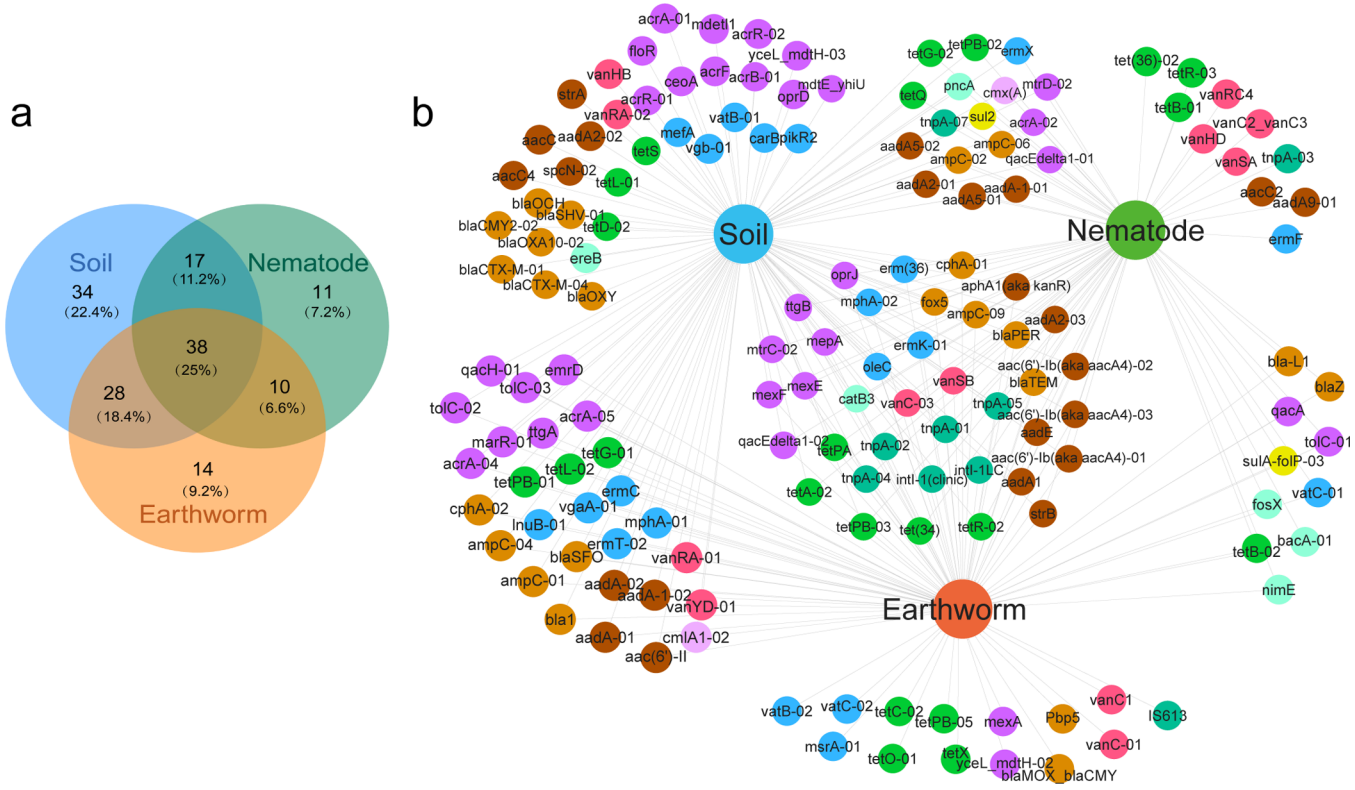

Figure 2. (a) Venn diagram showing the number and percent of shared ARGs in soil, nematodes, and earthworm guts. (b) Bipartite network analysis indicating the shared ARGs and MGEs between soil, earthworm guts, and nematodes.

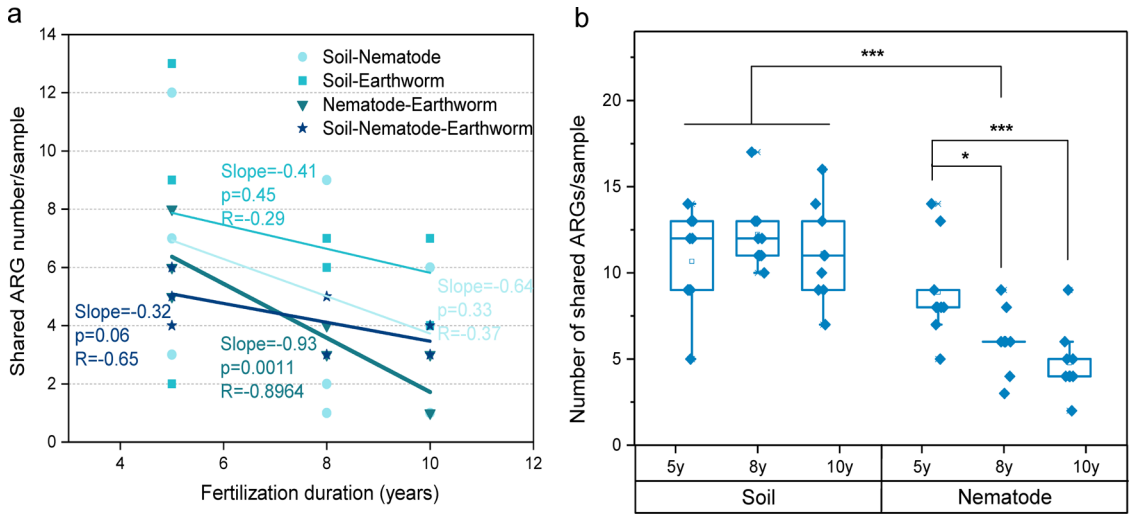

Figure 3. (a) Linear regression analysis of duration of fertilization and the number of shared ARGs for various combinations of soil, nematodes, and earthworm guts. The regression coefficients are represented by the slope of the line, and the linear dependences were depicted by Pearson correlation "*R*". (b) Number of ARGs shared across soil, nematodes, and earthworm guts in soil and nematode microbiome under different fertilization durations (5y, 8y, and 10y). Significance is annotated by "*" and "***", indicating $p < 0.05$ and $p < 0.001$ (ANOVA, Duncan test and T-test), respectively.

fertilization) and inorganic fertilizer (8-year) treatments but not the 10-year fertilization treatment. Fertilization had no significant effect on ARGs in the nematode and earthworm gut microbiome with the exception of a short-term mixed fertilizer application (5 years) on earthworm guts (Figure 1c, $p < 0.05$) compared to the control treatment. Compared to the control, the diversity of ARGs in the earthworm gut microbiota reduced after mixed fertilizer application (Figure S1, $p < 0.05$), and that in soil microbiota decreased with fertilization duration (Figure S1, $p < 0.05$). Relative abundance of ARGs and MGEs in the respective soil, nematode, and earthworm gut microbiome did not significantly change with fertilization (Figure S2a,c).

ARG profiles for soil, nematodes, and earthworm guts were influenced by fertilization duration (Figure S3, PERMANOVA test, $p < 0.05$) rather than fertilization treatment (Figure S3, PERMANOVA test, $p > 0.05$). The first two PCs in PCoAs using soil, nematodes, and earthworm guts explained 45.4,

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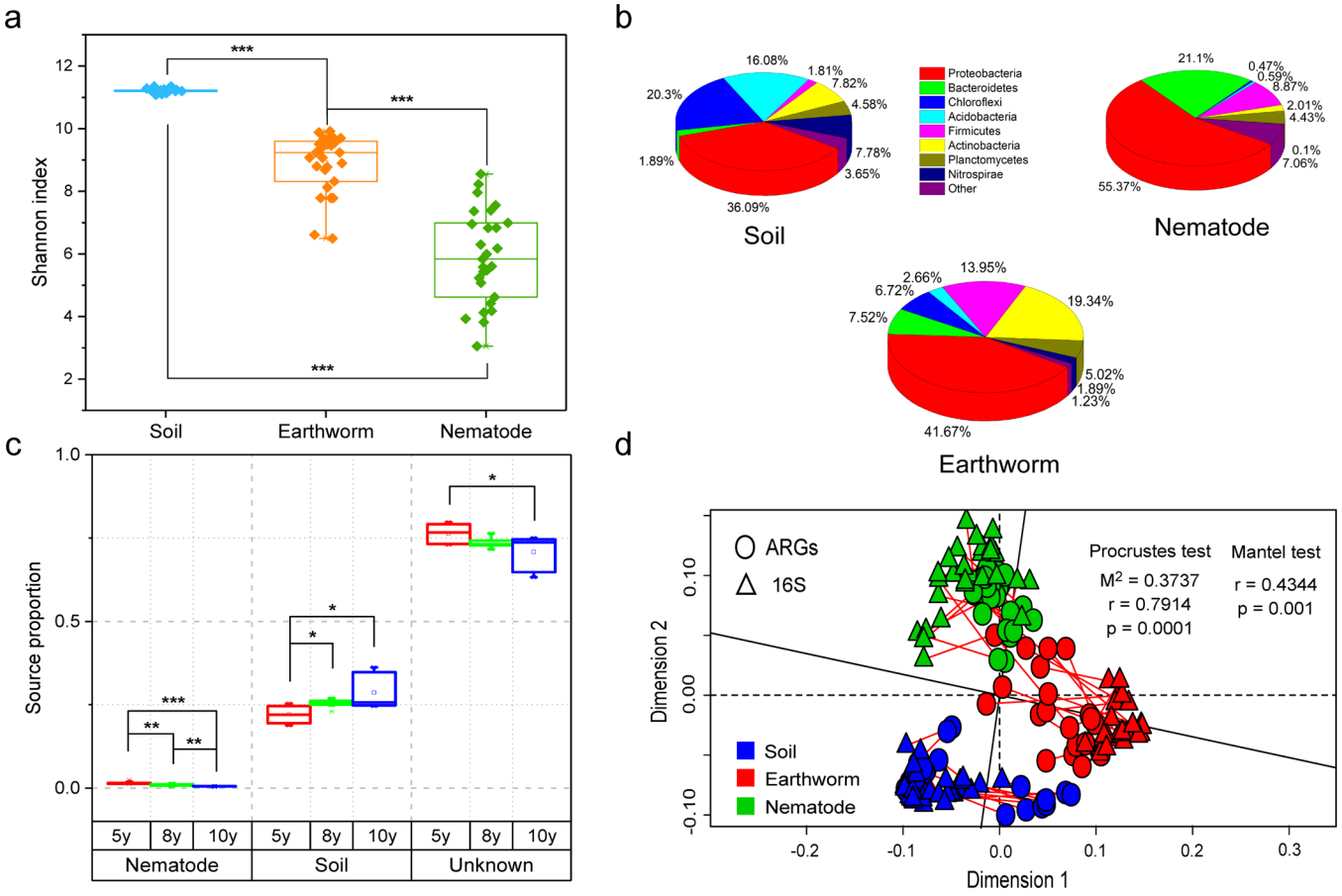

Figure 4. (a) Shannon index (mean ± SD, n = 27) of soil, nematode, and earthworm gut microbiome. The significant difference is denoted by "***", which represents $p < 0.001$ (ANOVA, Duncan test). (b) Mean percentage of each bacterial phylum (n = 27) in the soil, nematode, and earthworm gut microbiome. (c) FEAST estimations of source contribution to the sink, that is, gut microbiota of earthworm under different fertilization durations on the basis of a Bayesian classifier. Sources: nematode and soil microbiome at 5-, 8-, and 10-year fertilization time. Significant differences are denoted by "*", "**", and "***", which represent $p < 0.05$, 0.01, and 0.001, respectively (ANOVA, Duncan test). (d) Procrustes analysis between a shared microbial community (16S rRNA gene OTU data) and shared ARG profiles in soil, nematodes, and earthworm guts based on Bray–Curtis dissimilarity metrics (Procrustes sum of square M^2 = 0.3737, r = 0.7914, p = 0.0001, 9999 free permutations).

36.0, and 33.7% (PC1) and 23.3, 17.1, and 13.2% (PC2) of the variance, respectively (Figure S3). The number of MGEs detected from soil microbiota samples increased on average after 8-year fertilization from 1 to 3 (Figure S2d, $p < 0.05$), while the number of ARGs in soil decreased (from 28 to 21), and nematode (from 12 to 7) and earthworm gut microbiome (from 16 to 10) significantly decreased with fertilization duration ($p < 0.05$; nematode, $p < 0.01$, 10-year fertilization; Figure S2b). Ignoring the sample source, the number of ARGs was negatively related to duration of fertilization (Figure 1d, $p < 0.05$), with the strongest effect on earthworm guts (Figure 1d, coefficient = −1.53, $p < 0.001$, and Pearson's r = −0.620).

Shared ARGs between Soil, Nematodes, and Earthworm Guts. A total of 38 ARGs were shared between soil, nematodes, and earthworm guts (Figure 2a), which was greater than the number of ARGs in soil, nematodes, or earthworm guts individually or in combination. A bipartite network consisted of nodes divided into two types (sample sources and ARGs), where every edge connects a node of sample sources to one of the ARGs, highlighting shared ARGs between different sample sources. Shared ARGs represented seven antibiotic categories and MGEs (Figure 2b), with MGEs, aminoglycoside, and multidrug accounting for >55% of those recorded. Of those MGEs recorded, 2 integrase genes (*intI-1LC*, *intI-1*(*clinic*)) and 4 transposase genes (*tnpA-01*, *tnpA-02*, *tnpA-04*, and *tnpA-05*) comprised 67% of the 9 MGEs detected in the study (Figure 2b). Of the shared genes, the relative abundance of *intI-1*(*clinic*), *oprJ*, *mexF*, *blaTEM*, *tnpA-04*, and *tet*(*34*) was greatest (Figure S4). Of all combinations of shared ARGs examined, only those shared between nematodes and earthworm guts significantly decreased with fertilization duration (Figure 3a, coefficient = −0.93, p = 0.0011, and Pearson's r = −0.8964). More ARGs were shared in soil than in nematode samples (Figure 3b, $p < 0.001$). In contrast, fertilization duration had no effect on the ARG number in soil but led to a significant decrease in nematodes (Figure 3b, $p < 0.05$). Dominant genes driving the shifts between nematodes and earthworm guts included *mexF*, *ttgB*, and *aadA2-03* (5-year fertilization) and *vanSB* and *aac*(*6′*)*-Ib*(*aka aacA4*)*-01* (8-year fertilization), whereas there were no dominant genes after 10-year fertilization (Figure S5).

Microbial Community Composition and Structure. Soil had the greatest microbial diversity followed by earthworm guts and nematodes (Figure 4a, $p < 0.001$). PCoA showed a clear separation between samples (Figure S6, PERMANOVA test, $p < 0.001$). Bacterial taxa from soil, nematodes, and

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earthworm guts were also distinct. The most abundant phyla were Proteobacteria (36.1%), Chloroflexi (20.3%), and Acidobacteria (16.1%) in soil; Proteobacteria (55.4%) and Bacteroidetes (21.1%) in nematodes; and Proteobacteria (41.7%), Actinobacteria (19.3%), and Firmicutes (14.0%) in earthworm guts (Figure 4b).

FEAST analysis, a Bayesian approach measuring the respective similarities between a target microbial community and potential source environments and modeling the sink as a mixture of potential sources, was used to identify the origin of taxa and quantify the fraction of soil and nematode microbiome (sources) in earthworm gut microbiota (sink).[49] Earthworm gut OTUs (22.1%) were derived from soil, but only 1.6% was nematode-derived with the remainder (76.3%) being from unknown sources under the 5-year fertilization (Figure 4c). Earthworm gut OTUs originating from nematodes ranged from 1.6 to 0.5% with fertilization duration ($p < 0.01$), and the proportion of the soil source significantly increased to 28.7% under 10-year fertilization ($p < 0.05$).

Correlation Analysis and Co-occurrence Patterns between Bacterial Taxa and ARGs. The 38 shared ARGs including MGEs (Figure 2) and 21 shared OTUs (Figure S7) were selected for Procrustes analysis. A significant correlation was observed between microbiome and ARG profiles (Figure 4d, $M^2 = 0.3737$, $r = 0.7914$, $p = 0.0001$, 9999 free permutations). Network analysis revealed a strong correlation between bacteria and ARGs ($\rho > 0.4$, $p < 0.001$), with 41 nodes and 90 edges (Figure S8). Nodes were constituted of 8 ARGs (*aphA1*(*aka kanR*), *blaTEM*, *cphA-01*, *intI-1*(*clinic*), *mexF*, *oprJ*, *tet*(*34*), and *vanC-03*) and 33 bacteria. Bacterial taxa were mostly affiliated to Proteobacteria and Firmicutes. Bacillaceae, Microbacteriaceae, Rhodobacteraceae, Solibacteraceae, and MND1 (Betaproteobacteria) were correlated with *intI-1*(*clinic*) ($p < 0.001$). Genes *tet*(*34*) and *oprJ*, which are resistant to tetracycline and multidrug, had a strong correlation with multiple bacteria ($\rho > 0.6$, $p < 0.001$).

Relationships between Soil Properties, Microbiome, MGEs, and ARGs in Earthworm Gut Microbiota. Soil properties except ammonium nitrogen exhibited a significant change with fertilization duration as shown by linear regression analysis (Figure S9). For example, moisture content and available phosphorus (coefficient < 0, $p < 0.001$, and Pearson's $r < -0.6$) were negatively correlated to fertilization duration. Meanwhile, pH, organic matter, nitrate nitrogen, total nitrogen (coefficient > 0, $p < 0.001$, and Pearson's $r > 0.6$), and total phosphorus (coefficient > 0, $p < 0.01$, and Pearson's r = 0.5) were positively correlated with fertilization duration. RDA (Table S1) revealed that ARGs in earthworm gut microbiota were significantly correlated to pH ($p < 0.05$), ammonium nitrogen ($p < 0.01$), and available phosphorus ($p < 0.05$).

Drivers of ARGs shift in earthworm gut microbiota were assessed by SEMs (Figure 5). The shift of ARGs in the earthworm gut microbiota had a significant positive direct correlation with MGEs in the earthworm gut microbiota ($\lambda = 0.662$, $p < 0.01$) and a combination of both positive and negative indirect effects of the microbial diversity through MGEs ($\lambda = -0.372$, $p < 0.05$), fertilization treatment through MGEs ($\lambda = -0.376$, $p < 0.05$), or fertilization treatment through microbial diversity ($\lambda = 0.524$, $p < 0.01$).

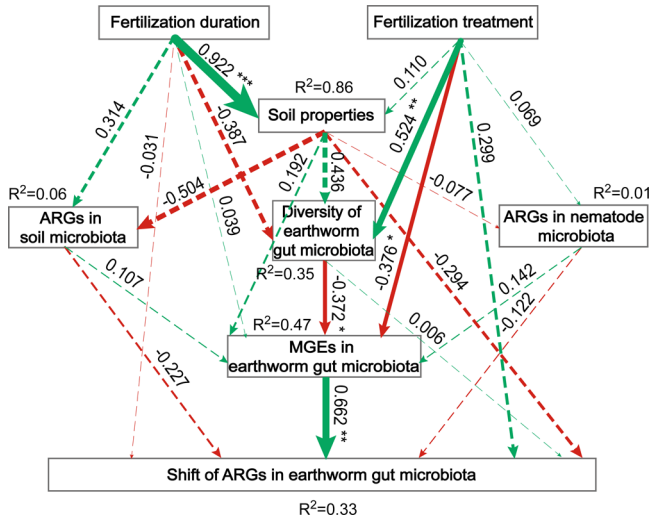

Figure 5. Structural equation models (SEMs) showing the effects of fertilization duration, fertilization treatment, soil properties, diversity of the earthworm gut microbiota, ARGs in the soil microbiota, ARGs in the nematode microbiota, and MGEs in the earthworm gut microbiota on the changes in ARG composition in earthworm gut microbiota. Solid and dashed arrows denote, respectively, significant and nonsignificant relationships, and the width of the arrows represents the strength of the path coefficients (numbers adjacent to the arrows). Green and red arrows represent positive and negative relationships, respectively. R^2 values indicate the proportion of explained variance. Significance levels are distinguished by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. The hypothetical model fits the data well: low chi-square ($x^2 = 1.485$), nonsignificant probability level ($p = 0.960$), degrees of freedom = 6, high goodness-of-fit index (GFI = 0.986), low Akaike information criteria (AIC = 61.485), and low root-mean-square errors of approximation (RMSEA < 0.001).

DISCUSSION

Fate of ARGs in Nematodes and Earthworm Guts. In this study, we found differences in the distribution of ARGs between soil, nematodes, and earthworm guts despite samples being sourced from an identical system. Application of mixed fertilizers significantly increased the number of ARGs in soil after 5- and 8-year fertilization but not 10-year fertilization. In contrast, the number of ARGs associated with nematodes and earthworm guts remained comparatively stable. This could be due to the reported moderating effect of the microenvironments in the intestines of earthworms and nematodes.[17,51] Although the relative abundance of ARGs increased in both nematodes ($p < 0.001$) and earthworms ($p < 0.05$), consistent with previous studies in pigs,[52] mice,[53] honeybees,[54] earthworms,[12] collembolans,[55] and nematodes,[11] the level of ARG enrichment in nematodes and earthworms was comparatively different. The potential difference in ARG dynamics between nematodes and earthworm guts may have led to a reduction in ARGs over time, which offers a potential means to mitigate ARGs added by organic fertilizers. Similar observations have been obtained in the housefly gut during vermicomposting of swine manure.[20] It is possible that the earthworm gut may filter out certain microbes ingested from soil due to the selective pressure imposed by the unique gut environmental properties,[30,51] forming a simpler niche than the surroundings and causing a reduction of potential ARG hosts. Furthermore, a number of bacteria carrying ARGs can be killed by digestive enzymes (e.g., chitinases, lipases, and cellulases) secreted into earthworm intestines[30,56] and subsequently assimilated. The reduced number and increased abundance of ARGs in nematodes and earthworm guts lead to the enrichment of just a few types of ARGs, potentially offering a means to

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predict which ARGs are likely to ultimately pose a potential risk along the food chain.

Given the strong correlation with bacteria, the distinct fates of ARGs between nematodes and earthworm guts may have been driven by selection through either differing microbial community composition or the unique gut microenvironment.[21] Actinobacteria and Firmicutes dominated in earthworm guts, whereas Bacteroidetes was the most abundant in nematodes. Actinobacteria produce antibiotics,[55] and Firmicutes are regarded as symbionts with hosts,[57] which may explain the higher diversity of bacteria in earthworm guts than those in nematodes. Additionally, many bacteria in Bacteroidetes are or easily become pathogens,[58] so the accumulation of ARGs in nematode microbiota may enhance the occurrence and dissemination of resistant pathogenic bacteria in soil ecosystems.

Transfer of ARGs along a Potential Soil Food Chain. FEAST analysis indicated that nematode microbiota could act as a limited source (up to 1.6% in this study) to the earthworm gut microbiota. In this study, shared ARGs and MGEs among soil, nematode, and earthworm samples contributed a lot to the detected ARGs and MGEs, which indicated the transfer potential of ARGs along the soil-nematode-earthworm food chain. ARGs can be transferred between nonpathogens and pathogens via MGEs (e.g., *intI* and *tnpA*) based on horizontal gene transfer,[59] and high-density microbiota in guts provide the place of transfer and increase the transfer frequency.[60] Thus, transfer of ARGs between bacteria within soil fauna gut microbiome was not unexpected. The transfer of ARGs between nematodes and earthworms, while significant, was small. With the majority of the earthworm gut microbiota derived from unknown sources, it is clear that multiple sources, including soil and soil fauna, are important to maintain the functioning of earthworm gut microbiota. Thus, it is necessary to explore the multiple factors and sources controlling the fate of ARGs in the earthworm gut microbiota.

In this study, fertilization duration not only significantly altered the distribution of ARGs (Figure 2) but also reduced the diversity of ARGs in soil (Figure S1) and the number of ARGs in nematodes and earthworm guts, especially earthworm guts (Figure 1d). Moreover, the transfer of ARGs was shown to be attenuated over time along this short food chain, particularly within the nematode-earthworm system. A close connection between shared ARGs and OTUs indicated that ARGs will likely exhibit a similar change with OTUs. The shared ARGs in the soil microbiota did not alter with fertilization duration, and those in nematode microbiota significantly decreased. In addition, earthworms may produce an eliminating mechanism under long-term fertilization stress[61] so that it could mitigate ARGs enriched from soil and nematodes. These account for the removal of ARGs in earthworm gut microbiota with fertilization duration.

Soil Properties, Bacterial Community, and MGEs Influencing the Spread of ARGs. Soil properties can drive the shift of ARG profiles in soil and soil biota.[5,12] Our study demonstrated that pH, ammonium nitrogen, and available phosphorus significantly affected ARGs in earthworm gut microbiota, which was consistent with the previous studies.[15,60] Earthworm guts typically maintain a relatively neutral pH homeostasis.[30] A study in Germany showed that a high concentration of ammonium, a near-neutral pH, and anoxia are a part of factors greatly enhancing anaerobic activities of earthworm guts.[62] Thus, ammonium nitrogen and available phosphorus ingested into earthworm guts can enhance the anabolism of gut microbiota,[30] accelerating removal of ARGs, which explains the decline in the ARG number in earthworm gut microbiota over fertilization time.

In this study, ARG composition was found to be correlated with bacterial community composition consistent with a previous long-term fertilization study in ARGs of earthworm gut microbiota.[12] A strong correlation between genes (*tet*(*34*), *oprJ*) and many bacterial taxa highlighted the prevalence and mobility of *tet*(*34*) (resistance to tetracycline) and *oprJ* (resistance to multidrug) genes in the system. The ubiquitous antibiotic groups tetracycline and multidrug ingested by soil fauna can generate a selective pressure prone to resistance to these antibiotics.[63]

We also found that ARGs of aminoglycoside and multidrug and MGEs were shared in the studied short food chain. MGEs are known to play an important role in the dispersal of ARGs and can potentially enhance the enrichment of ARGs in manure-fertilized soil via horizontal transfer.[64,65] Previous studies have shown that bacteria could harbor and express different exogenous ARGs through MGEs, especially integrons.[66,67] The class 1 integron gene is usually considered as a marker for pollution due to the close connection with many antibiotic/heavy metal resistance genes, rapid response to environmental pressures, and the xenogenetic "clinical" forms of *intI1* imposed by human activities.[66] In the present study, the integron gene *intI-1*(*clinic*) was highly abundant and significantly linked to five bacterial families. Additionally, MGEs have the greatest contribution to the shift of ARGs in earthworm gut microbiota. These MGEs are important because *intI1* and *tnpA* are linked to genes conferring resistance to multiple antibiotics and heavy metals, drive the gene horizontal transfer, and are involved in the emergence and spread of ARGs through the soil food web.[68] These results demonstrate that fertilization might cause a potential risk of ARG spread and pollution to the soil ecosystem.

In summary, we found a reduction in ARGs over time in both nematodes and earthworm guts. Soil properties, microbial community, and MGEs were identified as the main factors contributing to the shift of ARGs in earthworm gut microbiota. There were still some shortcomings in our study. For example, nematodes were hand-picked based on similar size and morphology under a dissecting microscope rather than identification according to species.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c03893.

(Methods) 16S rRNA amplicon sequencing, HT-qPCR for ARG analysis, and construction of structural equation models (SEMs); (Table S1) RDA analysis of soil properties on the ARG profiles in the earthworm gut microbiota; (Figure S1) Shannon index of ARGs in soil, nematode, and earthworm gut microbiome under different fertilization durations; (Figure S2) relative abundance (copies/16S rRNA gene) (a,c) and number (b,d) of ARGs and MGEs per sample in soil, nematodes, and earthworm guts; (Figure S3) PCoA of ARGs in soil (a), nematodes (b), and earthworm guts (c); (Figure S4) relative abundance of shared ARGs in the soil, nematode, and earthworm gut microbiome; (Figure S5)

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network analysis of the shared ARG types in soil, nematodes, and earthworm guts; (Figure S6) PCoA of soil, nematode, and earthworm gut microbiome; (Figure S7) Venn diagram in soil, nematodes, and earthworm guts; (Figure S8) co-occurrence network analysis of shared ARGs and shared bacteria; (Figure S9) linear regression analysis between soil properties and fertilization duration (PDF)

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Notes

The authors declare no competing financial interest.

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